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Prevalence of *Listeria* spp. and characterization of *Listeria monocytogenes* isolated from food products in Tetouan, Morocco

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Abstract

A total of 1096 food samples were purchased in Tetouan, North-Western of Morocco, to examine the presence of *Listeria* spp. Eighty (7.3%) of the tested samples were found positive for the presence of *Listeria* spp., while *L. monocytogenes* was detected in 16 (1.5%) samples. *L. monocytogenes* isolates belonged to clonal complexes CC2 (PCR serogroup IVb) and CC199 (PCR serogroup IIa) and to 8 different combined AscI/ApaI pulsed-field gel electrophoresis profiles. Core genome multilocus sequence typing (cgMLST) allowed to distinguish 12 different cgMLST types and revealed the presence of 3 clusters of closely-related isolates from different samples, suggesting the existence of common sources of contamination. Isolates showed no resistance to the reference antibiotics used for the treatment of listeriosis. This study underlines the circulation of *L. monocytogenes* strains of clonal complex CC2 in Morocco and highlights the importance of microbiological surveillance in order to minimize consumers' exposure to this foodborne pathogen.

Keywords: *Listeria*; Morocco; PCR-serogrouping; cgMLST.

1. Introduction

Listeria are Gram-positive ubiquitous bacteria, widely distributed in different environments and areas (Hamon et al., 2006). There are currently seventeen species in the genus *Listeria* grouped into *Listeria sensu stricto* (*L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. marthii*) and *Listeria* sensu lato (*L. fleischmannii, L. grayi, L. rocourtiae, L. weihenstephanensis, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis, L. booriae, L. newyorkensis*) (Orsi & Wiedmann, 2016). Among these species, *L. monocytogenes* is the causative agent of listeriosis, one of the most severe foodborne infections (Maertens de Noordhout et al., 2014). *L. monocytogenes* can resist disinfectants, form biofilms and survive or multiply under extreme physicochemical characteristics, such as dry environments, different temperatures, a wide range of pH and high salt concentrations. All these conditions promote the survival and proliferation of *L. monocytogenes* in a large variety of food matrices including unpasteurized dairy products, meat products, seafood, vegetables, and in food industry environments. The contamination of food products with this pathogen can occur during production, packaging, transport, and storage (Carpentier & Cerf, 2011). Human listeriosis can manifest as septicemia, meningoencephalitis, abortion or neonatal infection with high case fatality rate (20e30%) even in the absence of resistance to reference antibiotic treatment (Charlier et al., 2017; Morvan et al., 2010). In Morocco, the incidence of human listeriosis remains unknown due to the absence of epidemiological surveillance (Cohen et al., 2006; Maertens de Noordhout et al., 2014), and so far only one case of a neonatal listeriosis has been reported (Benabdejlil et al., 2015; Benomar et al., 2000). Despite the lack of surveillance for *Listeria* infections in Morocco, several studies reporting the prevalence of *L. monocytogenes* in food have been performed in the center of the country to estimate consumers' exposure to this pathogen. In Casablanca, for instance, *L. monocytogenes* was present in 14.4% of red meat products collected in 1998 (Kriem et al., 1998), absent in 156 red meat and offal samples collected between 2002 and 2004 (Cohen et al., 2006), and present in 2.3% of 426 poultry and red meat samples collected in 2008 (Ennaji et al., 2008). A study in the city of Rabat showed increasing prevalence of *L. monocytogenes* in various foodstuffs ranging from 0.8% in 2009 to 4.1% in 2011, with the highest prevalence (13.8%) in poultry products in 2010 (El Habib et al., 2014). Another study has reported a prevalence of *L. monocytogenes* of 3.7% in chicken meat samples collected in Rabat in 2011e2012 (Khallaf et al., 2016). In the Kenitra region, *L. monocytogenes* was detected in 0.8%

of raw milk samples (Hadrya et al., 2012), whereas in the region of Fez, the prevalence of *L. monocytogenes* was 5.9% in raw milk and traditional dairy products (El Marnissi et al., 2013). Nevertheless, the prevalence of *L. monocytogenes* in food items in Northern Morocco remains unknown. Thus, the aims of this study were: i) to evaluate the presence of *L. monocytogenes* and other *Listeria* spp. in different food matrices collected in Tetouan (North-Western of Morocco), ii) to characterize the antibiotic susceptibility profiles of *L. monocytogenes* isolates, and iii) to type *L. monocytogenes* isolates by PCR serogrouping, pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and core genome MLST (cgMLST).

2. Materials and methods

2.1. Sample collection and bacteria isolation

A total of 1096 food samples, including dairy products (*n*=404), bovine meat products (*n*=258), pastry (*n*=162), salads (*n*=143), poultry meat products (*n*=103), chickpea flour cooked with eggs sold in the street (*n*=20) and mayonnaises (*n*=6), were collected from January 2009 to August 2015. The samples were obtained from different food outlets in Tetouan (North-Western of Morocco). All samples were immediately transported to the laboratory at 5 ± 3 °C and were microbiologically examined within 1-2 h after sampling. Isolation and identification of *Listeria* spp. were performed according to the official method for the detection of *L. monocytogenes* (ISO, 1996). Biochemical identification was performed using the API- *Listeria* system (bioMérieux, Marcy l'Etoile, France) (Bille et al., 1992). *Listeria* strains were stored at -80 °C in Brain Heart Infusion (Biokar, Beauvais, France) with 20% glycerol until further use.

2.2. PCR serogrouping

PCR serogrouping was determined by multiplex PCR targeting *lmo0737*, ORF2819, ORF2110, *lmo1118*, and *prs* gene regions, using primers and conditions as previously described by Doumith et al. (2004). PCR products were resolved on a 2% agarose gel containing 0.5 mg/ml of ethidium bromide (Eurobio, Courtaboeuf, France) and documented using the Bio-Rad Gel Doc 2000™ imaging system (Bio-Rad Laboratories, Milan, Italy). Electrophoresis profiles were interpreted according to Doumith et al. (2004) and Leclercq et al. (2011).

2.3. PFGE AscI/ApaI typing

L. monocytogenes PFGE fingerprints were obtained with the restriction enzymes *Asc*I (New England BioLabs, Massachusetts, Ipswich, USA) and *Apa*I (MBI Fermentas, Burlington, Canada), as previously described by Graves & Swaminathan (2001) and Martin et al. (2006). The combined *Asc*I/*Apa*I PFGE pulsotypes were analyzed using BioNumerics software version 6.6 (Applied Maths, Kortrijk, Belgium). Bands automatically assigned by the computer were checked visually and corrected manually when necessary. Cluster analysis of the individual or combined PFGE pulsotypes was performed using the unweighted pair group method with average linkage (UPGMA) method. The similarities of the banding patterns were estimated based on Dice's coefficient (Dice, 1945). The discriminatory power was determined by

calculating the discrimination index (D) based on Simpson's index of diversity (Hunter Gaston, 1988). Two PFGE patterns were considered to be similar if they differed by less than two bands (Martin et al., 2006).

2.4. MLST and cgMLST molecular typing

L. monocytogenes genome sequences were obtained after DNA extraction (DNeasy Blood Tissue kit, Qiagen, Denmark), library preparation (Nextera XT DNA Sample kit, Illumina, California, USA) and sequencing using NextSeq 500 (2 x 150 bp) platform (Illumina, California, USA), according to the manufacturer's protocol. Draft assemblies were obtained using CLC Assembly Cell 4.3.0. (Qiagen, Hilden, Germany), as previously described (Moura et al., 2017). MLST and cgMLST profiles were obtained from genomic data as described previously (Moura et al., 2016; Ragon et al., 2008). cgMLST types were defined using the profile similarity cut-off of 99.6% (i.e. isolates sharing 7 or less allelic differences on 1748 alleles were considered the same type), as previously described (Moura et al., 2016). cgMLST clusters were defined as groups of isolates sharing the same cgMLST type.

2.5. Antimicrobial susceptibility testing

L. monocytogenes isolates were tested for their antimicrobial susceptibility using the agar disc diffusion method following the guidelines of the French committee on antimicrobial susceptibility testing (French Society of Microbiology [SFM], 2016; Morvan et al., 2010). Inoculum concentration was standardized by turbidimetry (Densimat photometer, bioMérieux). Mueller-Hinton agar plates (bioRad, Marne-la-Coquette, France) were inoculated with 1.0-2.0 x 10^8 cfu/ml and incubated at 35 °C for 24 h. The following discs (Oxoid Ltd., Hampshire, United Kingdom) of antimicrobial agents were used in this study: amikacin (30 mg), amoxicillin (25 mg), ampicillin (10 mg), cefotaxime (30 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), clindamycin (2 mg), erythromycin (15 mg), fosfomycin (50 mg), fusidic acid (10 mg), gentamicin (15 mg), imipenem (10 mg), kanamycin (30 mg), levofloxacin (5 mg), lincomycin (15 mg), moxifloxacin (5 mg), nalidixic acid (30 mg), penicillin G (6 mg), rifampicin (30 mg), streptomycin (10 mg), sulfametoxazole (200 mg), tetracycline (30 mg), trimethoprim (5 mg), tobramycin (30 mg), and vancomycin (30 mg). The diameters of growth inhibition zones were measured with Scan 4000 (Interscience, Saint Nom, France). Strains were classified as susceptible, intermediately resistant (reduced susceptibility), or resistant by using the

breakpoints recommended by the French Committee on antimicrobial susceptibility testing (French Society of Microbiology, 2016). *Streptococcus pneumoniae* ATCC 49619 and *Escherichia coli* ATCC 25922 were used as the quality control strains (French Society of Microbiology, 2016).

3. Results

3.1. Prevalence of Listeria spp. and L. monocytogenes

The distributions of the different *Listeria* species per type of sample are presented in Table 1. *Listeria* spp. isolates were detected in 80 out of 1096 (7.3%) food products. These belonged to *L. innocua* (57 samples; 5.2%), *L. monocytogenes* (16 samples; 1.5%), *L. welshimeri* (4 samples; 0.4%) and *L. seeligeri* (3 samples; 0.3%) (Table 1). None of the samples were simultaneously contaminated by different *Listeria* species. The sampling plan used did not allow to clarify the prevalence of *Listeria* per type of food per year. Among the 80 positive samples, the highest prevalence of *Listeria* spp. were found in mayonnaise (33.3%) and chickpea flour cooked with eggs sold in the street (25%), followed by poultry meat (14.6%) and bovine meat (12.8%) products. Lower prevalence was observed in pastries (4.9%), dairy products (3.2%) and salads (2.8%). *L. monocytogenes* was predominantly isolated from mayonnaise (16.7%), pastries (3.1%) and bovine meat products (2.7%) (especially mortadella), and to a lesser extent in dairy products (0.7%) (raw milk, traditional whey) (Tables 1 and 2).

3.2 *Molecular typing*

L. monocytogenes isolates (*n*=16) belonged mainly to PCR serogroup IVb (87.5%) and only two isolates from raw milk belonged to PCR serogroup IIa (12.5%). All *L. innocua*, *L. welshimeri* and *L. seeligeri* isolates belonged to PCR serotype L, which is the characteristic serogroup of other species than *L. monocytogenes*. PFGE subtyping of *L. monocytogenes* isolates showed 8 different PFGE combined *Asc*I/*Apa*I types (Fig. 1), with three different PFGE clusters (named I to III). *L. monocytogenes* isolates were assigned to MLST clonal complexes CC2 (*n*=14; 87.5%) and CC199 (*n*=2; 12.5%). Genome-based typing revealed 12 different cgMLST types and 3 clusters of isolates sharing the same cgMLST type (CT2833, CT2841 and CT2002) (Table 2, Fig. 2).

3.3 Antimicrobial resistance

L. monocytogenes isolates showed natural resistance to cefotaxime, sulfonamide, nalidixic acid, fosfomycine and lincosamide. Two isolates (CLIP 2016/00835 and CLIP 2016/00836,

isolated from rawmilk) were resistant to tetracycline, due to the presence of *tetM* gene as revealed by genome analysis (data not shown). Resistance to penicillin G, chloramphenicol, rifampicin, streptomycin, vancomycin, fusidic acid, trimethoprim, levofloxacin, moxifloxacin, ciprofloxacin, erythromycin, amikacin, kanamycin, amoxicillin, ampicillin, gentamicin, imipenem and tobramycin was not observed (data not shown).

4. Discussion

This study focused on the detection and characterization of food-associated *L. monocytogenes* isolated in the North region of Morocco, in order to evaluate the burden of this pathogen in different food products. Overall, *Listeria* spp. were detected in 7.3% samples, being *L. innocua* (5.2%) the most prevalent species, followed by *L. monocytogenes* (1.5%). Nevertheless, one cannot exclude that the presence of *L. innocua* in the samples analyzed could inhibit the growth of *L. monocytogenes* initially present in the samples, resulting in false-negative detection of *L. monocytogenes* (Carvalheira et al., 2010). Prevalence values of *L. monocytogenes* previously reported for other regions of Morocco (Ennaji et al., 2008; Hadrya et al., 2012) were in agreement with those found in the present study, but were different from those reported in others studies (Cohen et al., 2006; El Habib et al., 2014; Khallaf et al., 2016). In this study, all *L. monocytogenes* found in bovine meat products were recovered from mortadella. The prevalence of *L. monocytogenes* in mortadella (7.0% among the 100 mortadella samples analyzed) was similar ($p=0.6$, χ^2 test) to the one reported in Egypt (Reda et al., 2016). Furthermore, several studies that examined the prevalence of *L. monocytogenes* in food (except for mayonnaise) from different Mediterranean countries reported higher prevalence values compared to our results: 5.8% in raw milk (Boubendir et al., 2011); 14.5% in salad samples (Effimia, 2015), 45.0% in poultry meat (El Mali et al., 2015) and 10.0% in pastries (Di Pinto et al., 2010). The differences in the prevalence of *L. monocytogenes* could be attributed to the geographical differences, sampling strategy, hygienic conditions and microbiological analysis methods. Reference treatment for listeriosis is based on the administration of a synergistic combination of aminopenicillin (ampicillin or amoxicillin) with or without gentamicin. For patients allergic to those antibiotics, trimethoprim-sulfamethoxazole is recommended as alternative treatment (Hof, 2004). *L. monocytogenes* isolated during our study showed no resistance to the reference treatment antibiotics. These

results were in agreement with those reported in previous studies (Charlier et al., 2017; Morvan et al., 2010). *L. monocytogenes* isolates belonged mostly to PCR serogroup IVb (87.5%), belonging to phylogenetic lineage I. Isolates from lineage I have been reported as overrepresented among human isolates, while those from lineage II are overrepresented among food isolates (Maury et al., 2016). PCR serogroup IVb has been associated to the majority of clinical strains causing severe human infections (Maury et al., 2016). Moreover, during 2010-2012, the majority of deaths in Europe were linked to the two most common PCR serogroups IIa and IVb (ECDC, 2015). In the present study, the strains belonging to the first PFGE cluster (I) corresponded to *L. monocytogenes* isolates of PCR serogroup IVb isolated from mortadella, pastries, traditional whey, and mayonnaise collected from urban areas (Fig. 1). All strains belonging to the second PFGE cluster (II) were isolates of PCR serogroup IIa from raw milk in a rural area, where livestock production is artisanal. The third PFGE cluster (III) comprised of isolates of PCR serogroup IVb, isolated from pastries, sampled in 2012 in the same site, as in cluster (I), and from two street vendors (I-1 and I-2), located in the ancient city, where the hygienic conditions are unfavorable. In addition, it is noteworthy that these last street vendors bought their pastries from the same producer. *L. monocytogenes* isolates belonged to MLST clonal complexes CC2 and CC199. Maury et al. (2016) have shown that CC2 is one of the clonal complexes significantly associated with clinical isolates in France and specifically with materno-neonatal infections. Moreover, Chenal-Francisque et al. (2011) have shown that the frequent clone CC2 was found in more than 30 countries and was globally distributed. The circulation of this hypervirulent clonal complex CC2 highlights the importance for epidemiological surveillance of listeriosis, especially the maternal-neonatal forms of the disease (Leclercq et al., 2014; Maertens de Noordhout et al., 2014). Genome-based typing allowed to detect three clusters of *L. monocytogenes* isolates sharing the same cgMLST type (Fig. 2), suggesting the existence of common contamination sources. CT2833 cluster was associated with pastry from the same place of sampling (street vendors) where the hygienic conditions were likely unfavorable, and around the same period, consistent with the suspicion of a possible cross-contamination with other sold foods, such as traditional milk products, a contamination in the production environment and/or in the raw materials used to produce pastry. CT2841 cluster was associated with mortadella imported from Spain which was grated for the preparation of sandwiches and with pastry from a different place and period. Mortadella of this cgMLST cluster were sampled from the same vendor and around the same

period, also in favor of a contamination due to a resident strain in the environment or in the raw materials used to produce this product. Isolates from cluster CT2002, as described for PFGE cluster (II), were originated from raw milk sold by local farmers on a local weekly market in a rural area, where livestock production is artisanal, which could involve animal contamination or poor hygiene during milking.

5. Conclusion

Listeria spp. were detected in different types of products from North of Morocco. *L. monocytogenes* isolates belonged primarily to PCR serogroups IVb and IIa and MLST clonal complexes CC2 and CC199. cgMLST analysis allowed to detect clusters of isolates likely sharing common sources of contamination. The current study was the first one to characterize *L. monocytogenes* strains from Morocco at the genomic level. Due to the lack a record of listeriosis cases and associated strains in Morocco, the public health impact of these results remains to be elucidated. This study highlights the importance of monitoring the dissemination of this pathogen in North Morocco and of reducing the exposition of the population, especially pregnant women, to *L. monocytogenes*.

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Table 1. Prevalence and biochemical identification of *Listeria sp.* in food samples in Tetouan (NW of Morocco) from 2009 to 2015.

Type of sample	No. samples analyzed	No. *Listeria*-positive samples (%)	*L. innocua* (%)	*L. monocytogenes* (%)	*L. welshimeri* (%)	*L. seeligeri* (%)
Dairy products	404	13 (3.2)	6 (46.2)	3 (23.1)	3 (23.1)	1 (7.7)
Meat products	258	33 (12.8)	24 (72.7)	7 (21.2)	0 (0)	2 (6.1)
Egg products	103	15 (14.6)	14 (93.3)	0 (0)	1 (6.7)	0 (0)
Pastry	162	8 (4.9)	3 (37.5)	5 (62.5)	0 (00)	0 (0)
Salads	149	6 (4.0)	5 (83.3)	1 (16.7)	0 (0)	0 (0)
Chickpea flour cooked with eggs sold in the street	20	5 (25.0)	5 (100)	0 (0)	0 (0)	0 (00)
Total	**1,096**	**80 (7.3)**	**57 (71.3)**	**16 (20)**	**4 (5)**	**3 (3.8)**

Table 2. *Listeria monocytogenes* isolated in food samples in Tetouan (NW of Morocco) from 2009 to 2015.

CLIP Number[a]	Original laboratory number	Sample	Sampling date	Sampling anonymized origin – Street vendor	Phylogenetic lineage	PCR serogroup[b]	MLST Clonal Complex[d]	PFGE *Asc*I/*Apa*I Cluster[c]	cgMLST type[d]
2016/00804	18lm	Traditional whey	28/04/2009	G	I	IVb	CC2	I	CT2839
2016/00806	1Pal	Mayonnaise	01/07/2009	H	I	IVb	CC2	I	CT2843
2016/00835	Sara1	Raw milk	05/01/2011	J-1	II	IIa	CC199	II	CT2002
2016/00836	Sara2	Raw milk	05/01/2011	J-2	II	IIa	CC199	II	CT2002
2016/00851	4P	Pastry	01/05/2012	I-1	I	IVb	CC2	III	CT2840
2016/00852	7MF	Pastry	08/05/2012	I-2	I	IVb	CC2	III	CT2834
2016/00853	3P	Pastry	01/05/2012	I-1	I	IVb	CC2	I	CT2833
2016/00854	6MF	Pastry	08/05/2012	I-2	I	IVb	CC2	III	CT2833
2016/00875[f]	100/1	Mortadella	01/07/2013	E-3	I	IVb	CC2	I	CT2841
2016/00876	66/11	Mortadella	03/06/2013	A-4	I	IVb	CC2	I	CT2838
2016/00877	73S	Mortadella	08/06/2013	B	I	IVb	CC2	I	CT2835
2016/00878	93/2	Mortadella	27/06/2013	C-5	I	IVb	CC2	I	CT2836
2016/00879	69/1	Mortadella	06/06/2013	D-4	I	IVb	CC2	I	CT2842
2016/00880	93/1	Mortadella	27/06/2013	C-5	I	IVb	CC2	I	CT2837

2016/00882 [f]	100/2	Mortadella	01/07/2013	E-3	I	IVb	CC2	I	CT2841
2016/00886	E4BMH	Pastry	09/06/2015	F	I	IVb	CC2	I	CT2841

a, CLIP: Collection *Listeria* Institut Pasteur
b, according to Doumith *et al.* (2004)
c according to Graves and Swaminathan (2001)
d, according to Moura *et al.* (2016)
e, according to Ragon *et al.* (2008)
f, same sample

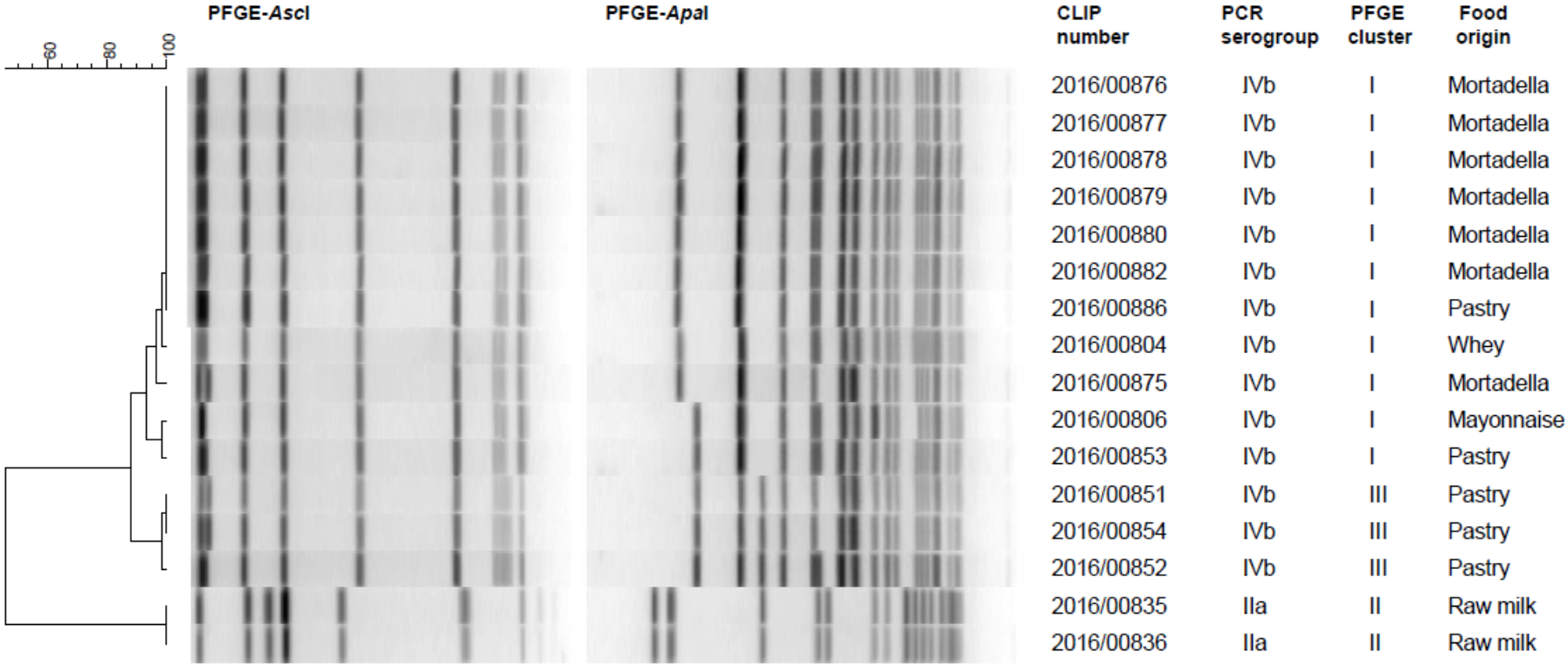

Figure 1. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Pulsed-field Gel Electrophoresis (PFGE) profiles with AscI and ApaI endonuclease enzymes of *L. monocytogenes* isolated from food items.

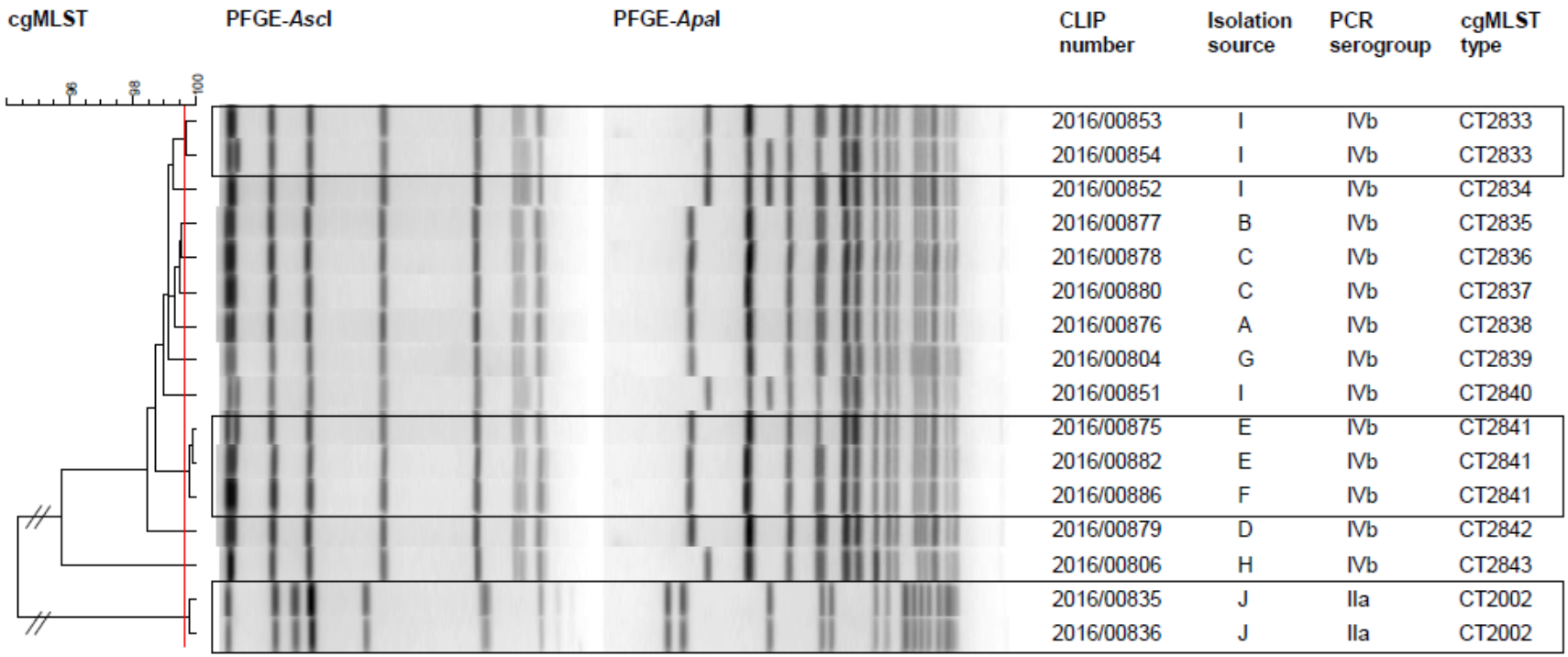

Figure 2. Single linkage dendrogram based on *L. monocytogenes* cgMLST profiles from genoserotype IVb. The vertical bar indicates the cgMLST type cut-off of 99.600% (Moura et al., 2016). Horizontal boxes highlight clusters of isolates belonging to the same cgMLST type.